IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of :
Praveen SHARMA et al. :
Serial No. 09/429,003 : Examiner: J.C. Einsmann
Filed: October 29, 1999 : Group Art Unit: 1655
For: METHOD OF PREPARING :
A STANDARD DIAGNOSTIC GENE :
TRANSCRIPT PATTERN :

DECLARATION UNDER RULE 132

Commissioner of Patents
and Trademarks
Washington, D.C. 20231

I, Praveen Sharma, an Indian citizen of Waldemar Thranes gate 62D, N-0173, Oslo, Norway; and

I, Anders Lönneborg, a Swedish citizen of Feltspatveien 14, N-1430, Aas, Norway,

declare as follows:

1. We are inventors on the present application. We have reviewed the Office Action dated 29 January 2002 which issued on the above application, wherein the Examiner raised objections under 35 U.S.C. 112, first paragraph, that the specification does not enable performance of the invention as claimed.

2. To demonstrate that the invention as claimed may be put into practice in accordance with the specification, we provide details of experiments which we have conducted in our laboratory which identify differentially expressed transcripts in various diseased samples and have used these transcripts as probes to classify test samples as being derived from patients with the disease in question or from patients without that disease. The samples which have been examined are peripheral blood samples taken distant to the site of disease. The disease states which have been investigated are Alzheimer's disease and breast cancer. The experiments which have

been conducted are described in the following paragraphs.

Alzheimer's disease example

3. The following experiment was conducted to identify transcripts, or their related products, which could be used for probes to diagnose for Alzheimer's disease. The successful use of those probes for such a diagnosis is also described.

4. In this experiment 7 patients diagnosed with Alzheimer's Disease at the Memory Clinic at Ullevål University Hospital were used in the trial. The patients were confirmed as having Alzheimer's disease based on the following criteria:

* A standardized interview with a care-giver using IQCODE, an ADL scale and a scale measuring behaviour of the patient (Green scale).
* Neuropsychological evaluation using MMSE, Clock drawing test, Trailmaking test A and B (TMT A and B), Kendrick object learning test (visual memory test), part of the Wechsler battery and Benton test.
* A psychiatric evaluation using scales for detection of depression, MADRS for interviewing the patient and Cornell scale for interviewing the care-giver.
* A physical examination.
* Laboratory tests of blood samples to rule out other diseases.
* CT scan of the brain.
* SPECT of the brain.

The mean age of the patients was 72.3 with an age range of 69-76. The mean MMSE score was 22.0 (the maximum score attainable being 30).

5. Six age-matched individuals without diagnosed Alzheimer's disease were used as a control. All had been tested with MMSE and had a minimum score of 28 (mean: 28.4). The mean age of the normal control group was 73.0 and the age range 66-81. A sample from a 16-year old individual, with a consequent minimal chance of having Alzheimer's disease, was also included.

6. Whole blood was obtained from the arms of the Alzheimer's disease and control (normal) group patients. In both cases 10ml of whole blood was collected in tubes containing EDTA and stored immediately at -80°C until used for mRNA extraction.

7. mRNA was isolated from the blood of the Alzheimer's disease patients and from the control group donors and labelled during reverse transcription in the presence of α^{32}P-dATP, yielding a labelled first strand cDNA.

8. The resulting labelled first strand cDNA of the normal and diseased samples was hybridized, separately, to 758 random cDNA clones, picked from a cDNA library from whole blood of 550 healthy individuals without knowledge of the gene sequence of the random cDNA clones, which were immobilized on a solid support.

9. The amount of labelled first strand cDNA binding to the immobilized clone probes was assessed and quantified using a PhosphoImager to determine the relative signal for each probe and used to generate a gene expression data set. The generated data set was then normalized to take account of differences in the probe intensities resulting from the experimental conditions. The data set was then analysed to identify clones within the 758 random clones which were informative for distinguishing between normal and disease samples.

10. In this experiment 170 genes were found to be differently expressed (Group mean > 1.3 fold) in the cells from blood of patients with Alzheimer's disease versus normal individuals without Alzheimer's disease. For illustrative purposes, 32 genes which show this differential expression are shown in Figure 1 in Annex 1.

11. To employ this finding diagnostically, the normalized data for the 7 control and 7 Alzheimer's disease samples hybridized to the transcript products of these 32 genes were examined further. The normalized data which provide the relative signal for each probe for each sample were manipulated using conventional statistical techniques known in the art at the priority date

(namely Unscrambler software) to generate a classification model. The results of this classification model is shown in Figure 2 (Annex 2) in which it will be seen that the expression pattern of these genes was able to classify individuals with or without Alzheimer's disease into distinct groups. In this Figure PC1 and PC2 indicate the 2 principal components statistically derived from the data which define the systematic variability present in the data. This allows each sample, and the data from each of the 32 probes to which the samples' cDNA was bound, to be represented on the classification model as a single point which is a projection of the sample onto the principal components - the score plot.

12. The ability of the general model (based on the 32 differently expressed genes) to correctly diagnose samples was determined by cross-validation. In this approach, the step of generating the classification model was performed as described above, but the data of a single sample (and its replicates if these are present) are omitted from the data used in that modelling process. The accuracy of the generated model is then determined by using that model to classify the omitted sample as belonging to the disease or non-disease class. This process is repeated for each sample to obtain information on the accuracy of diagnosis. The cross-validation results and details of the success of the diagnostic test are shown in Table 1 in Annex 3. Only a single false negative was diagnosed. No false positives were diagnosed. This illustrates the diagnostic value of examining relevant gene expression data sets which can be readily generated using a routinely obtained blood sample.

Breast cancer example

13. Similar experiments were conducted with samples from breast cancer patients. Whole blood was obtained from the arms of breast cancer patients and patients with benign tumours (Ullevål and Haukland hospitals in Norway). All of the patients with breast cancer had a malignant tumour of the breast (disease samples). Healthy blood was collected from the above two hospitals, or collected at a Health station at Ås, Norway or at DiaGenic AS, Norway, from the arms of female donors with no reported signs of

breast cancer. The blood from healthy individuals or with benign tumours comprise the normal samples. The blood was either collected in tubes containing EDTA (as described above for the Alzheimer's example) or was collected in PAXgene tubes and stored for 12-24 hours at room temperature before finally storing them at -80°C before use. Further details of the breast cancer and benign tumour patients from which blood was taken is provided in Table 2 in Annex 4.

14. Using the methods described above (except that hybridization to 1435 rather than 758 random cDNA clones was performed), 172 differently expressed genes (>1.3 fold) which were informative for distinguishing between normal and breast cancer patients were identified. For illustrative purposes, 25 genes which show this differential expression are shown in Figure 3 in Annex 5.

15. To employ this finding diagnostically, the normalized data for the control or cancer samples hybridized to the transcript products of these 25 genes were used to generate a calibration model as described in paragraph 11, above. The results of this classification model are shown in Figure 4 (Annex 6) in which it will be seen that the expression pattern of these genes was able to classify most women with breast cancer and women with no breast cancer into two distinct groups. Among those samples correctly classified were the three breast cancer samples from patients with Ductal Carcinoma In Situ (DCIS). In DCIS the lesion consists of a malignant population of epithelial cells and is confined by the basement membrane, i.e. the malignant cells are entirely located within the milk ducts and have not spread into any other tissue of the breast and have not initiated angiogenesis. The method also successfully identified patients with more clinically advanced stages of cancer.

16. The ability of the generated model (based on the 25 differently expressed genes) to correctly diagnose samples was determined by cross-validation as described in paragraph 12, above. The cross-validation results and details of the success of the diagnostic test are shown in Table 3 in Annex 7.

17. The above described experiments illustrate that cells obtained distant to the site of disease contain altered levels of gene expression in cells from normal relative to diseased patients. Those altered levels may be used to diagnose whether a patient suffers from a particular disorder.

18. Our experiments have furthermore revealed that our diagnostic test is not dependent upon transcripts reflecting an activated immune response. Thus the transcripts identified for diagnostic purposes need not correspond to genes such as interleukins or their receptors. Instead, genes that may be classified as house-keeping or metabolic function related genes, can be used for diagnostic purposes. Thus, the types of genes that may be used reflect the influence of other factors on the cells, ie. effects mediated without direct contact of the cells with disease cells, e.g. at the site of disease.

19. These results illustrate that the altered gene expression of cells in disease versus normal individuals, in samples obtained distant from the site of disease containing cells which have not contacted the site of disease, can be used diagnostically. It is our opinion that we have recognized a general phenomenon which would occur in all diseases, or stages thereof in view of the reaction of the entire body to that disease which will ultimately affect the gene expression of each cell within the body.

20. We further declare that all statements made herein of our own knowledge are true, and that all statements made on information and belief are believed to be true, and that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Codes, and that such wilful false statements may jeopardize the validity of the application and any patent issuing thereon.

Praveen Sharma 18 Nov, 2002

Praveen Sharma Date

18 Nov. 2002

Anders Lönneborg Date

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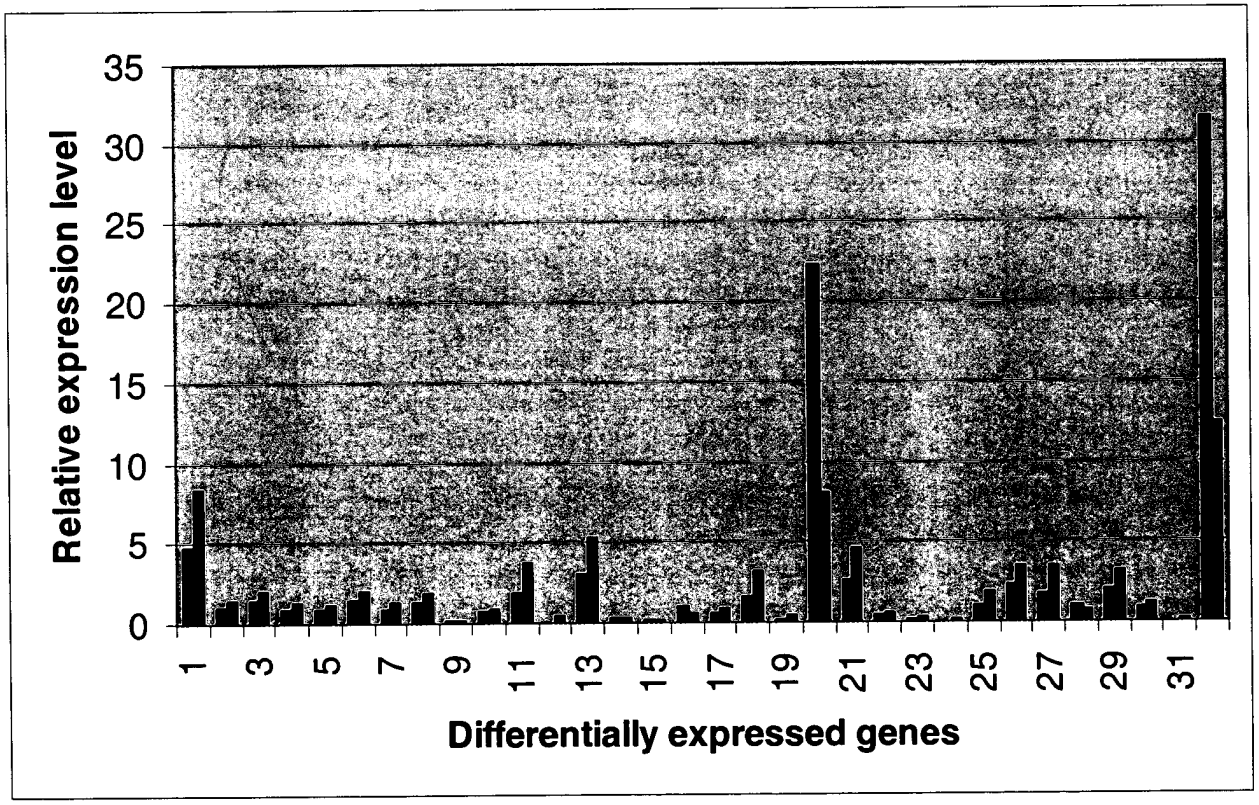
ANNEX 1

Figure 1

Mean expression level of 32 selected genes that are differentially expressed in whole blood of individuals with or without Alzheimer's disease

Red bars - Non-Alzheimer's disease
Blue bars – Alzheimer's disease



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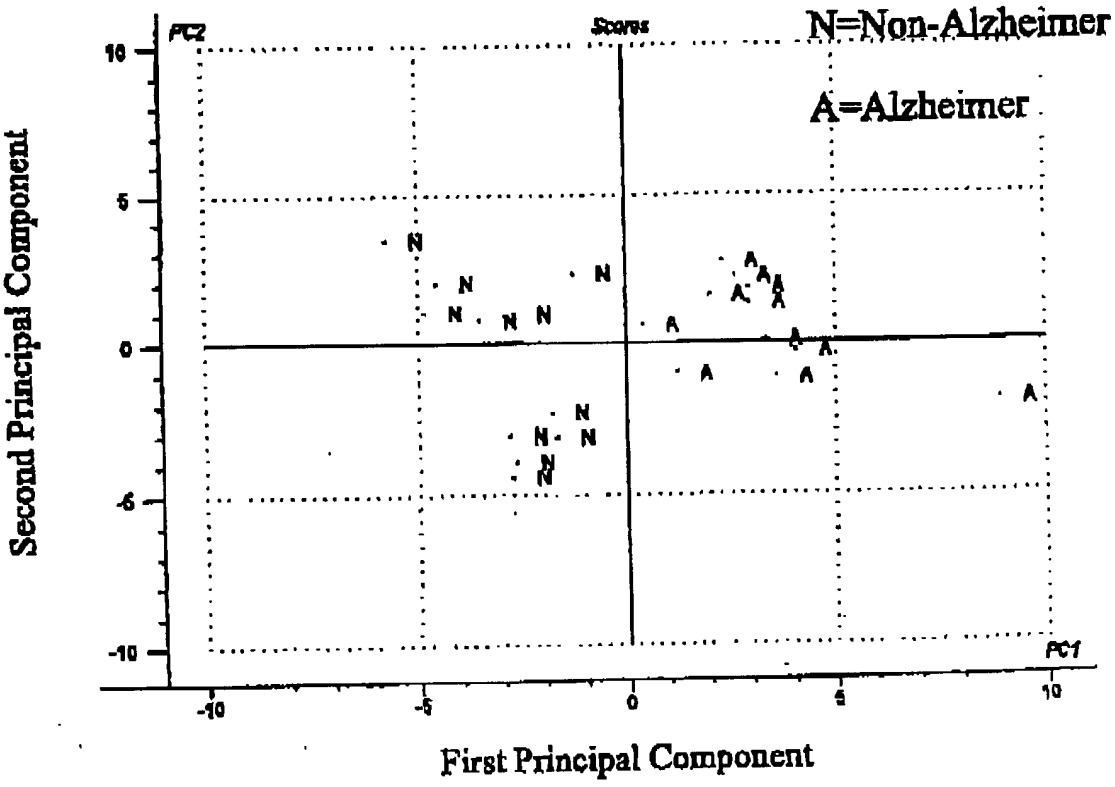
ANNEX 2

Figure 2

Projection of normal and Alzheimer's disease samples onto a classification model generated using the data of 32 different expressed genes (some samples were tested in duplicate or triplicate)

PC = principal components
N = Non-Alzheimer's disease
A = Alzheimer's disease



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ANNEX 3

TABLE 1 : Cross-validation and details of the success of the diagnostic test for Alzheimer's disease based on 32 differentially expressed genes

Validation Result

Total number of samples tested	14
Number of Alzheimer's disease samples tested	7
Number of Alzheimer's disease samples incorrectly diagnosed	1
Number of non-Alzheimer's disease samples tested	7
Number of non-Alzheimer's disease samples incorrectly diagnosed	0

Success of diagnostic test

Measure of performance	Description	%
Accuracy	Percentage of the total number of diagnoses that were correct	92.9
Sensitivity	Percentage of positive cases that were correctly identified	85.7
Specificity	Percentage of negative cases that were correctly diagnosed	100.0
False positive rate	Percentage of negative cases that were incorrectly classified as positive	0.0
False negative rate	Percentage of positive cases that were incorrectly classified as negative	14.3
Total error rate	Percentage of the total cases incorrectly diagnosed	7.1

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ANNEX 4

TABLE 2 : Details of the breast cancer and benign tumour patients

Samples

Diagnosis	No. of women
Normal/benign	34*
DCIS	3
Invasive Cancer	20

* From one woman, whole blood was collect at weeks 1, 2, 3, 4 and 5 following menstruation. Hence, the number of unique normal/benign samples tested in the experiment is 38.

Tumour size in women with breast cancer

Tumour size (mm)	No. of women
4-10	7
11-20	4
21-30	2
31-40	1
41-50	1
51-60	0
61-70	1
not known	7

Age distribution

Age	No. of women with breast cancer	No. of women without breast cancer
<40	0	8
40-49	3	15
50-59	2	11
60-69	10	1
>69	1	0
not known	4	2

Table 2 (contd.)

Other diseases/conditions present in the women tested

Disease/Condition	Number of women with disease
Diabetes	2
Asthma	1
Ulcerous colitis	1
Hypothyrosis	1
Crohn's disease	1
Fibromyalgia	1
Psoriasis	1
Allergies	20

Prior history of cancer in the women tested

Cancer type	No. of women
Breast	3
Colon	1
Stomach	1
Skin	1

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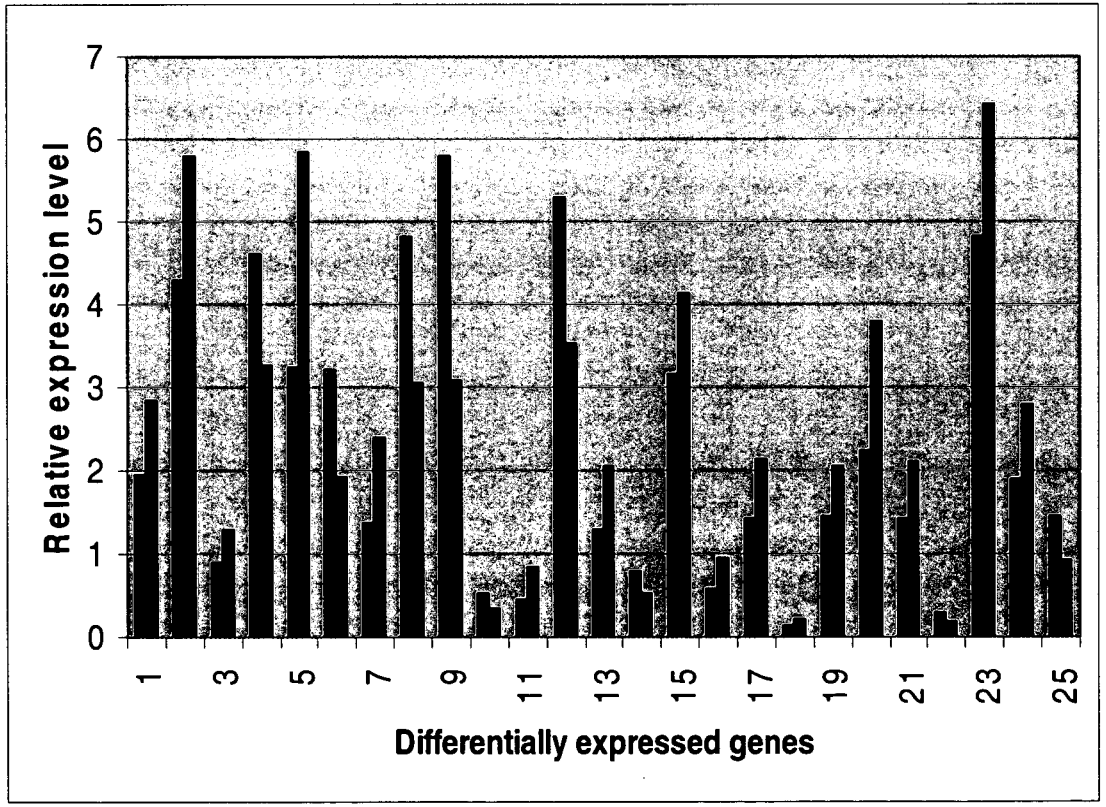
ANNEX 5

Figure 3

Mean expression level of 25 selected genes that are differentially expressed in whole blood of women with or without breast cancer

Red bars – Normal or benign
Blue bars – Breast Cancer



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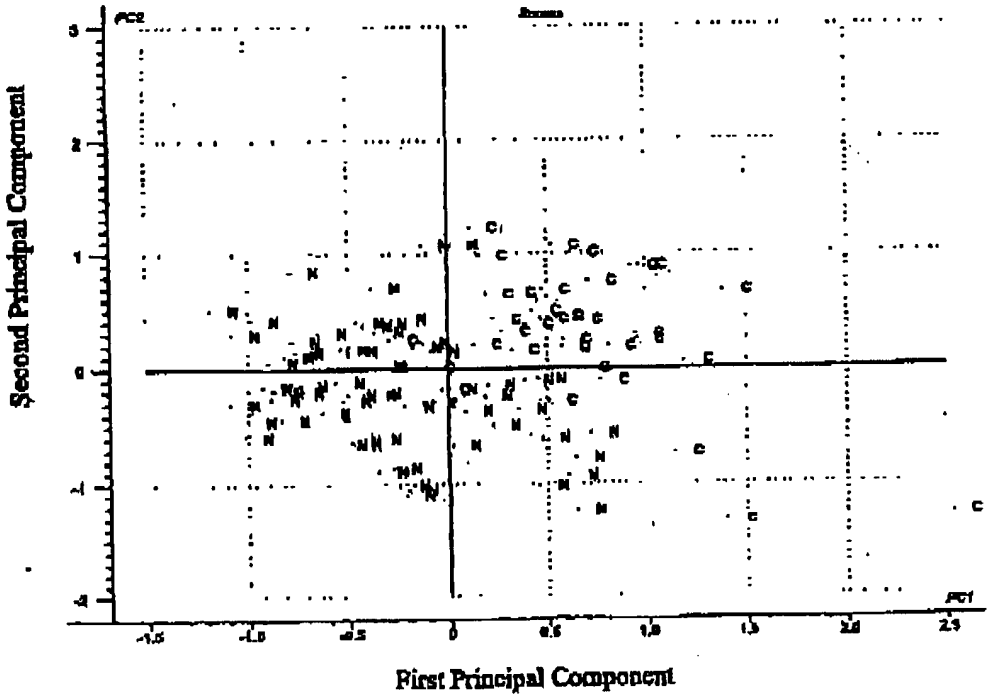
ANNEX 6

Figure 4

Projection of data from normal (including benign) and cancer samples onto a classification model generated using the data of 25 different expressed genes (some samples were tested in duplicate or triplicate)

PC = principal components
N = normal
C = cancer patient



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ANNEX 7

TABLE 3 : Cross-validation and details of the success of the diagnostic test for breast cancer based on 25 differentially expressed genes

Validation Result

Total number of samples tested	61
Number of cancer samples tested	23
Number of cancer samples incorrectly diagnosed	6
Number of non-cancer samples tested	38
Number of non-cancer samples incorrectly diagnosed	5

Success of diagnostic test

Measure of performance	Description	%
Accuracy	Percentage of the total number of diagnoses that were correct	82.0
Sensitivity	Percentage of positive cases that were correctly identified	73.9
Specificity	Percentage of negative cases that were correctly diagnosed	86.8
False positive rate	Percentage of negative cases that were incorrectly classified as positive	13.2
False negative rate	Percentage of positive cases that were incorrectly classified as negative	26.1
Total error rate	Percentage of the total cases incorrectly diagnosed	18.0